M2 channel: viruses serving mankind

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Abstract

M2 channel is a viral channel that is embedded in the envelope-membrane of the influenza A virus. It is a tetrameric protein consisting of four identical subunits of 97 amino acids forming a tetramer. The N-terminal domain is located on the outside of the virus envelope membrane and is important for incorporation of the channel in the membrane. The transmembrane domain, which traverses the lipid bilayer, contains a few key amino acids that are important for the functioning of the channel. The C-terminal domain is located on the cytoplasmic side of the envelope membrane and is important for the replication of the influenza virus. When the virus enters the host cell by endocytosis, the host cell tries to digest it by lowering the internal pH of the endosome. When the pH drops below 6.3, structural changes occur in M2 channel, it opens and allows only H^+ ions to enter into the viral envelope. As a result the RNA dissociates from the proteins bound to it. At the same time the haemagglutinin proteins, which are present in the viral envelope, induce the fusion of the viral membrane and the endosomal membrane of the host. As a result of that the viral RNA can enter the host cell and be expressed. Structure and functioning of M2 channel offers new tools to nanoscience, for example a drug delivery vehicle or a pH controller in chemical reactions.

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1. Introduction

1.1 What are channels?

All cells have membranes, which separate the inside of the cell from the external environment. These membranes consist of a lipid bilayer that is not permeable for ions. Even though the cell has its own content, it needs to get the necessary molecules inside or release the waste outside. In order to communicate in such a selective way with its environment, the cell uses membrane proteins, which are incorporated in the membrane. Two classes of these proteins can be distinguished: pumps and channels. *Pumps* use active transport to drive the uphill transport of ions or molecules. Energy input is needed for this process to take place. *Channels* use passive transport, which means that they enable molecules to flow in the direction of the concentration gradient through the membrane. The most important properties of channels are that they are highly selective for particular ions, they exist in open and closed state and the transitions between those states are regulated and the open states of channels spontaneously convert into inactivated states.[1a] There are already many examples of possible uses for these membrane proteins in nanotechnology for functions that are different than their natural one.[2,3] This requires the engineering of these proteins on the basis of the knowledge about their structure and functioning in nature. Therefore, let us start by looking at the most simple kind of these proteins, which are viral channels.

1.2 What are viral channels?

All viruses have a protein coat that protects their DNA or RNA. Some viruses also have an envelope, which surrounds the virus when it is outside a cell. Because this envelope consists of a lipid bilayer, proteins can be embedded in them like they are in membranes of other cells. Viral channels consist of short proteins of about 100 amino acids. Four different channels are found so far in enveloped viruses of eukaryotes (Table 1). It is suggested that the last three channels share similar roles and function in the life cycles of their viruses.[4] In this report, out of these four channels, the most extensively investigated M2 channel is going to be examined in more detail.

Table 1: Known viral channels.[5]

Virus	Channel
Influenza A	M2
Influenza B	NB
Influenza C	CM2
Human immunodeficiency virus type 1 (HIV-1)	Vpu

1.3 What is M2 channel?

The M2 channel consists of four identical subunits forming a tetramer. Each subunit consists of 97 amino acids. The N-terminal domain is short (21 amino acids) and is located on the outside of the virus, the transmembrane (TM) domain spans the lipid bilayer. It consists of 25 amino acids and connects the N- and the C-terminus of the protein. The C-terminal part is relatively longer (51 amino acids) and is located at the cytoplasmic side of the envelope.[4] A schematic drawing of M2 is shown in Figure 1.

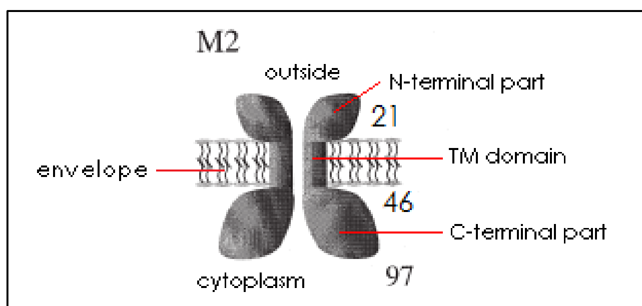

Figure 1: Schematic drawing of the M2 channel. Indicated are the N-terminal part at the outside of the virus, the transmembrane domain and the C-terminal part at the cytoplasmic side of the virus. The numbers indicate the position of amino acids in the protein.[5]

The N-terminal domain, which is on the extracellular side of the virus, is important for incorporation of the channel into the envelope. This part of the M2 protein is also a target for anti-viral drugs. Sera raised against this extracellular domain can passively induce protective immunity in mice. Broad-spectrum influenza vaccines, which enhance the immune response and prevent the virus to be lethal, have been designed to aim for this extracellular domain. However, when designed on the base of the human influenza strains, the antisera do not react with the N-terminal domain of M2 channel. Antibodies aimed at this N-terminal domain do not bind to the virus particle, but they do bind strongly to the virus-infected cells. This is probably due

to differences in the amino acid sequence between the recombinant vaccine and the challenge strain, which was observed in a study in swine.[6]

The tetrameric form of the channel is stabilized by non-covalent interactions in the transmembrane domain and by disulphide linkages in the N-terminal part. The TM-part is oriented in a helical structure and at pH 8.0 it has a 25° tilt. The most important amino acids present in the TM-domain are Histidine, which is the 37th amino acid of the protein (His37) and Tryptophan, the 41st amino acid (Trp41) (Fig 2). They are both hydrophilic and conserved in all known strains of influenza A viruses. Trp41 is located one helical turn from His37, so their side chains are pointing in the same direction into the inside of the channel. The side chain of His37 is called as an imidazole ring, which can be positively charged and the side chain of Trp41 is an indole ring, which has negative charges from the π-electrons. Because of their relative positions inside the channel, the side chains can easily interact with each other. Namely, a cation-π interaction occurs. This stabilizes the open form of the channel by partial neutralization of the positive charges, which are present on the His37 side chain in open form of the channel.[6]

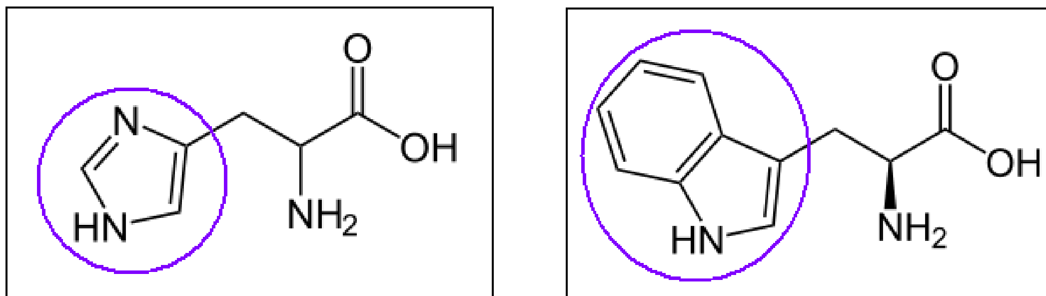

Figure 2: Structure of Histidine (left) and Tryptophan (right) as free amino acids, not incorporated in a protein. The circles in the left and right drawings indicate the imidazole and indole ring, respectively.

The C-terminal domain, which is on the cytoplasmic side of the virus, plays a role in the replication of the influenza virus and it is essential in the function of the M2 channel.[7] It binds to the matrix protein M1, which is the major internal structural component of the virion and plays an important role in the virus budding of Influenza A. By doing this it supports the assembly and production of infectious particles. The cytoplasmic tail also facilitates the efficient packaging of genome segments in the virions.[6]

1.4 What is the function of M2 channel in nature?

A schematic representation of the life cycle of the influenza A virus is shown in Figure 3. First step in the way to replication of the virus is binding of the glycoprotein haemagglutinin (HA), which is on the envelope of the influenza A virus, to the sialic acid-containing receptors on the surface of the host cell.[1a] This locks the two membranes together, so that the virus enters the host cell via receptor-mediated endocytosis (inlet Fig 3). The host cell tries to digest this intruder by acidifying the endosome. In response to this low pH (5-6) the structure of the HA protein changes. Part of the protein, called the fusion peptide, becomes exposed to the aqueous environment and because of its hydrophobic nature, it inserts itself into the endosomal membrane. The HA structure changes further and this pulls the viral and the endosomal membranes together, which eventually leads to fusion.[8]

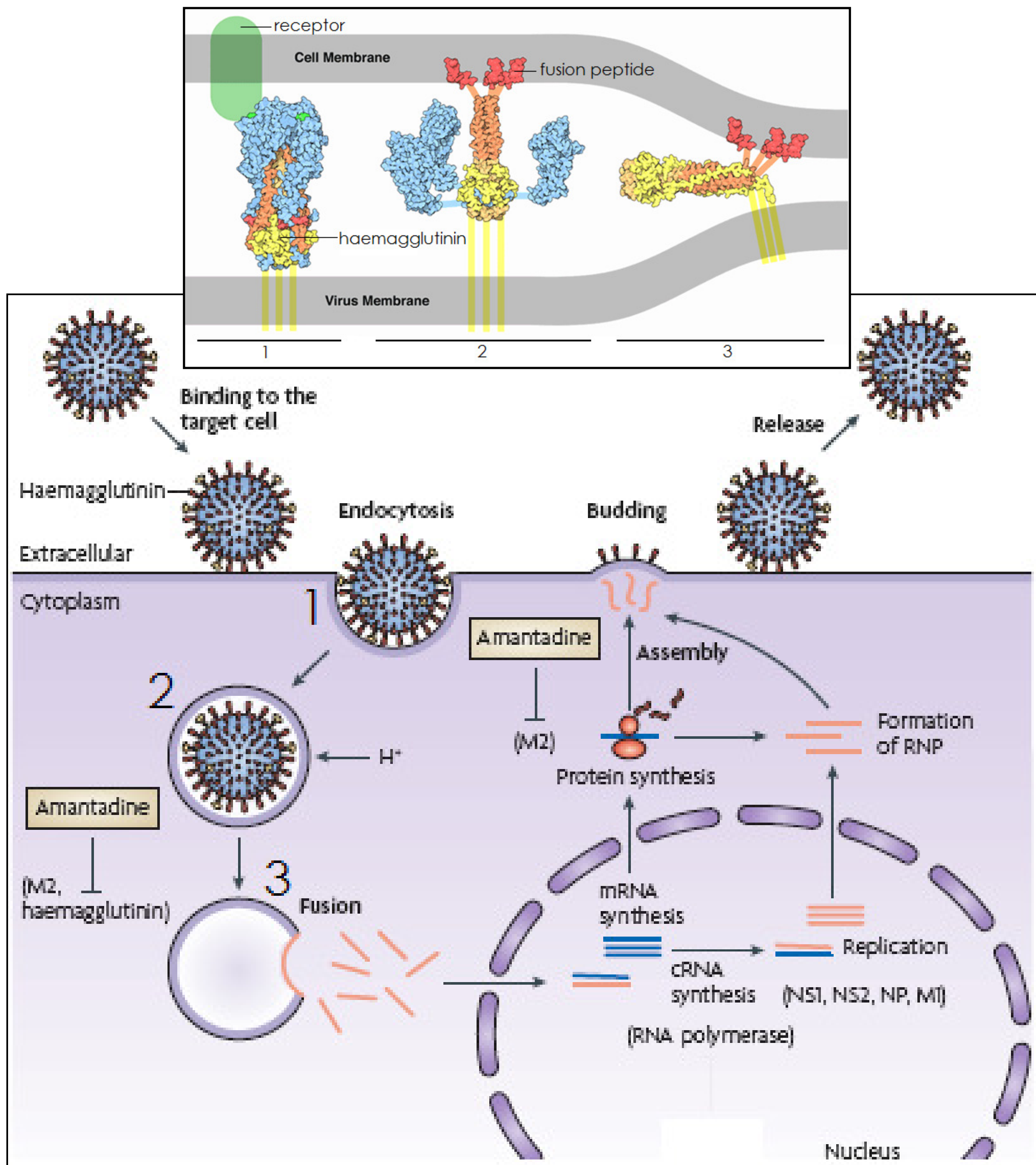

Figure 3: Life cycle of the Influenza A virus. The virus enters the host cell via endocytosis, the RNA is released and finds its way to the nucleus where it is replicated and a new virus particle is released.[9] *The numbers indicate the different steps corresponding to the different conformations of HA shown in the inlet. Inlet: Mechanism of the membrane fusion process induced by HA protein. 1) Binding of the HA protein to the sialic acid-containing receptor (green). 2) A pH-induced conformational change of HA, which liberates its hydrophobic fusion peptide (red) that will stick into the membrane. 3) The second structural change in HA, which pulls the two membranes together.*[8]

After fusion there is no other barrier between the inside of the virus and the cytoplasm of the host cell, so the genetic information is free to move towards the nucleus of the host cell. Prior to this membrane fusion, the M2 channel in the viral envelope opens in response to the low pH. The channel is proton-selective, so protons flow into the virus and the low pH induces the dissociation of the matrix protein M1 from viral ribonucleoprotein (vRNP). RNP denotes a complex of RNA with proteins bound to it (Fig 4).[6]

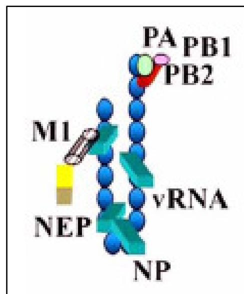

Figure 4: RNP complex. The viral RNA is associated with the nucleoprotein (NP) and three subunits of the viral polymerase complex (PA, PB1 and PB2). The nuclear export protein (NEP) is associated with RNP and the M1 protein is associated with the RNP and the viral envelope.[6]

Among the functions of RNP is transport and translation of mature mRNA.[10] The virus contains 8 molecules of single stranded RNA, which will be released into the cytoplasm of the host cell as free RNPs. These RNPs migrate to the nucleus of the host cell, where the transcription and replication of the viral RNA takes place, performed by vRNPs.[6] When the genome is expressed, new virus particles are formed and released to the outside of the host cell (Fig 3).

1.5 What is the mechanism of M2 channel at the molecular level?

In the closed state the His37 amino acids of the four different subunits are oriented with their imidazole side chains directed towards the inside of the channel. This way they occlude the pore. The Trp41 molecules form hydrophobic interactions with Leucine and Isoleucine side chains of the adjacent helix, thereby stabilizing the tetramer. Below pH 8.2 two of the four His37 are protonated, forming two +1 dimer species His-HisH^{+}. Within each dimer hydrogen bonds are formed between the imidazole and imidazolium. Two of the four helices are bend and create a small pore in the His37/Trp41 region. When the host cell acidifies the endosome, the pH is lowered and below pH 6.3 the third His37 residue is protonated. This leads to breaking of the 2-fold symmetry of the +1 dimers and activates the channel. The helices of the C-terminal half of the TM helix diverge towards the C-terminus, thereby creating an open cavity.[4]

Valine27, Alanine30 and Glycine34 form an aqueous pore together with His37 and Trp41. This is confirmed by hydrogen-deuterium exchange measurements, because those residues exchange more rapidly than residues located in the cytoplasmic domain.[7] It is found, using molecular dynamics simulations, that a wire of water is formed over which protons diffuse through the hydrogen-bond network of water (the so-called Grotthuss mechanism). However, this water wire is broken at the His37 constriction. Two models are proposed for the mechanism of the passage of a proton through the His37 tetramer: the "Gating model" and the "Shuttle model".[4]

The Gating model proposes that the third protonation of His37 at pH 6.3 opens the channel because of electrostatic repulsion. This way the water wire becomes continuous and the protons can hop along the water wire via this Grotthuss mechanism over the entire length of the channel.[4,5] An illustration of this mechanism is shown in Figure 5.

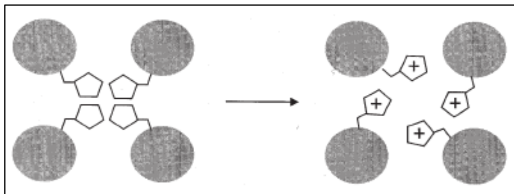

Figure 5: Proposed mechanism for the Gating model. The side chains of the four His37 residues are shown. When His37 is protonated, the side chains will be positively charged and because of electrostatic repulsion the channel is opened.[5]

The Shuttle model suggests that the imidazole nitrogen of His37, which faces the extracellular side of the membrane, binds one proton to form a biprotonated intermediate. The other nitrogen releases its proton on the intracellular side and thereby returns the imidazole to the neutral form. After this, the initial state of the molecule is recovered by tautomerization of the imidazole.[4,5] The mechanism is shown in Figure 6.

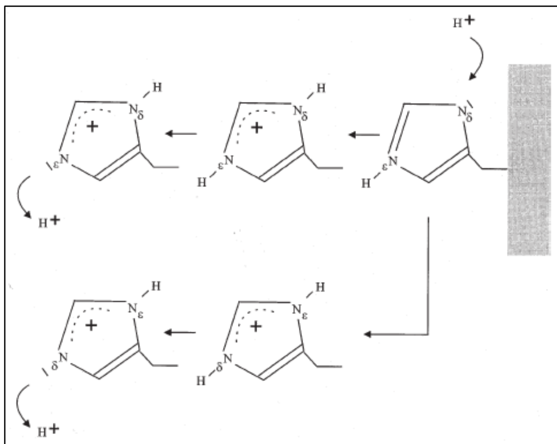

Figure 6: Proposed mechanism for the Shuttle model. One His37 side chain is shown. A proton is bound to one side of the ring and a positive charge occurs. To remove this charge, one proton is released from the other side of the ring.[5]

Proton transport can only take place if the channel is opened decently. In the described models, the channel is opened at the position of His37, but it is thought that His37 is the pH sensor (or selectivity filter) and Trp41 is actually the gate.[4,7,11] When His37 is protonated, the Trp41 side chains rotate towards the His37, because the formation of cation-π interactions favours this conformation. The negative charge of the indole π-electrons partially neutralizes the positive charge on the His37 molecules and thereby stabilizes the open form of the channel. The rotation of the Trp41 side chains leads to a conformation parallel to the axis of the pore. Because of the His37 side chains forming a barrier it is still impossible for most ions to flow through, but the small protons can.[7] The conformational change can be seen in Figure 7.

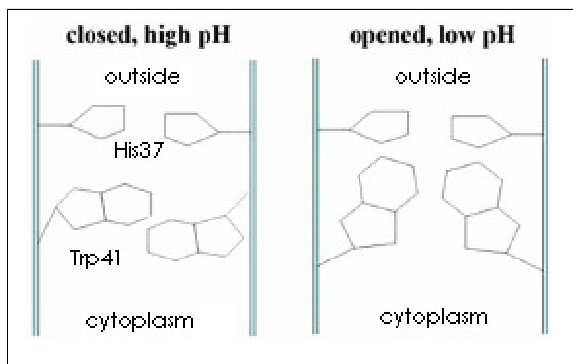

Figure 7: Mechanism of opening of the M2 channel. Only two of the four His37 and Trp41 residues are shown for clarity. When His37 is protonated, Trp41 rotates towards His37 and forms a cation-π interaction, thereby opening the channel.[6]

The opening or closing of the channel is only dependent on the pH on the outside of the virus. (pH_{out}). The channel is completely closed above pH 7.5 and open below pH 6.3. No outward current of H^+ is observed, which is probably a consequence of the bulky side chains of Trp41.[7,11]

It is found that the antiviral drug amantadine (Fig 8) inhibits the replication of the virus. This is a consequence of the inhibition of the M2 channel activity. It is suggested that amantadine acts from the outside of the aqueous pore and it interacts with the M2 channel in its tetrameric form and already in concentrations below 1 μM.[5] The adamantane group of the amantadine molecule, which is the bulky part of the molecule without the NH_2 group, is hydrophobic and therefore interacts with the side chains of the hydrophobic residues that line the pore. Perhaps also a hydrogen bond is created between the ammonium nitrogen of amantadine and the imidazole ring of His37, which will interrupt the cation-π interactions between His37 and Trp41 and prevent the channel from opening.[7]

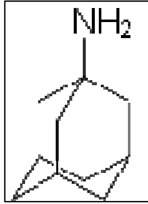

Figure 8: Structure of the amantadine molecule.

1.6 State of the art and prospects.

There is a relatively clear overview of what the M2 protein looks like and how it functions. It forms a tetramer with the transmembrane part in an α-helix form. It is a proton channel that plays an essential role in virus replication. The protonation of His37 and the rotation of Trp41 are essential for gating. Amantadine can block the channel and thereby prevent virus replication.[5] So far, the most information about the channel is found by doing experiments with M2 in detergents and micelles as mimics of lipid bilayers. Most data are observed for only the transmembrane part of the protein. It will be desirable to perform full-length protein studies, especially for understanding the function of the C-terminal and N-terminal parts.[4] It is found that parts of the cytoplasmic tail can be removed without losing its ion channel activity. Probably this C-terminal part plays a role in other processes that are involved in virus replication. An important question that remains is: How is it possible that the channel is able to let proton flow into the virion, while a positive potential should be building up on the inside?

2. Applications

2.1 Drug delivery

One of the applications of M2 channel in nanoscience is drug delivery. Methods for delivery of drugs that are used nowadays, like intravenous injection, oral dosage or suppositories, spread the drug throughout the entire body and this way it will also affect the healthy tissue. There are two consequences of these conventional drug delivery methods. First, the dose should be high enough to have a considerable effect and the second, there should be a proper balance between the toxicity of the drug and the therapeutic effect. In essence every drug is toxic, because most of the times it is not a compound that is present in the body in nature. However, it is the drug that is able to cure some particular disease. In conventional drug delivery methods only a small fraction of the drug will eventually arrive at the right spot, so quite a large amount of the drug has to be given to the patient. To overcome this problem, three stages are important. First the drug should be encapsulated into a carrier. The carrier prevents the interaction of the drug with its environment, until it arrives at the right place.[12] This would drag the equilibrium between the toxicity and the therapeutic effect of the drug towards the therapeutic effect. Less amount of the drug is needed, so that it will not be toxic and cause side effects, but still enough of the drug will arrive at the right place in the body to be able to cure. One of the most promising carriers are liposomes. A liposome is a small vesicle that consists of a lipid bilayer surrounding an aqueous environment. They are relatively easy to prepare and modify and to load with different kinds of drugs.

Once the drug is encapsulated in a proper carrier, the second important stage is the targeting. The drug carrier should be targeted to the specific place in the body, so that it will accumulate and the amount of locally released drug will be high enough to have a considerable effect. In case of liposomes used as carriers, this can be achieved by coating the liposomes with ligands or antibodies that direct them to the receptors in the target cells, such as cancer cells or tumors and inflammation sites.[13]

The last stage is the triggered release of the drug. After the carrier with the drug inside has arrived at the right place in the body, a trigger is needed to release the drug in that place. This trigger can be external or internal.[12]

However, even though all these three stages materialize, the next challenge in case of intracellular drug delivery is prevention of degradation of the drug by metabolic enzymes inside the cell. To prevent degradation, the drug should escape from the liposome as soon as it is taken up by the cell.

This is where the influenza A M2 channel becomes important for applications in nanoscience. The mechanism of influenza A to deliver its RNA into the host cell offers an alternative mechanism for intracellular drug delivery. Instead of the RNA, a drug can be put inside the virion and the virion can be released into the body. When the virion reaches a place with pH below 6.3, this triggers the virion to burst open and release the drug directly into the cytoplasm of the cell. The problem of degradation is circumvented, because the virus will release the drug immediately.

The method could be very advantageous especially for the delivery of small interfering RNA (siRNA). SiRNA is a class of small double-stranded RNA, about 20 to 25 nucleotides. It can influence the expression of particular genes inside the cell. The delivery of 'naked' siRNA is difficult, because the cellular permeability is low. Several methods have been tried to increase this permeability, but those are not so convenient for use *in vivo* applications. Another current limitation in the delivery of siRNA is its stability. SiRNA is readily degraded by RNases, which are present both intracellular and extracellular environments. [14] To overcome these problems, encapsulating the siRNA inside the virion might be a good solution. This way, the siRNA would not have to travel through the membrane. It would be taken up by cells themselves via endocytosis of the virus, hence there is no diffusion problem anymore. In addition it would be directly delivered from the virion into the cytoplasm. This way degradation of the siRNA would be prevented and it would not be necessary to chemically modify the siRNA.

Drug delivery is not limited with only intracellular delivery, it is also possible to deliver a drug around the cells, for example in the stomach or the duodenum. What is important in this case is that the opening of M2 channel should not be limited to a pH of 6.3, because the pH of gastric acid in the stomach is about 2.0 and the pH in the duodenum is about 5. In the case of the virus in its natural form and environment, His37 is the pH sensor and the virus will release its contents when triggered at a pH of 6.3 or lower. To deliver drugs to for example the stomach, the histidine residue should be replaced with a residue that is sensitive to lower pH. So the system could be modified for delivering drugs to the right places. For example the amino acids aspartic or glutamic acid (Fig 9) can be used instead of histidine, because they both have a pKa of 4.1.[1b] In

this case the channel would open at much lower pH than in the case of histidine. However, one has to keep in mind that it should still be possible for the alternative residue to form a cation-π, or any other convenient, interaction. In the case of aspartic and glutamic acid, above a pH of 4.1 they will be in their deprotonated form, this means with a negative charge. This negative charge would repel the negatively charged indole ring of tryptophan and the channel would be closed. When the acid groups are protonated, the –OH group can form an interaction with the tryptophan and the channel would open.

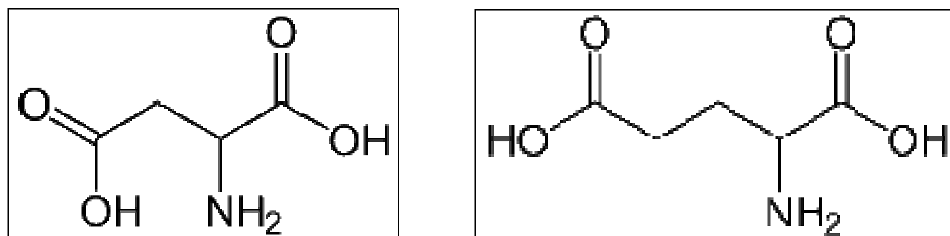

Figure 9: Structures of aspartic acid (left) and glutamic acid (right) as free amino acids, not incorporated in a protein.

Instead of searching for amino acids, which have the right pKa, it would be more favourable to have complete freedom in regulating the pH at which the channel will open. This can be achieved by first replacing Histidine at the 37th position in the protein with the amino acid cysteine (Fig 10) in order to generate a specific anchor point in the protein. This amino acid has a thiol side chain. Second, a specific base molecule with a low pKa could be designed and synthesized. This molecule should also have at one end a thiol-reactive group. Now this artificial pH-sensor could be covalently attached to the cysteine side chain by forming a disulfide bridge with the free thiol group of the cysteine in the protein. It is convenient to use a base with a low pKa, because this way it will be in its neutral form at higher pH and it will get protonated in an acidic environment. When it is protonated, it would form a cation-π interaction with Trp41 in the same way as His37. An example of such a compound is aniline (Fig 11). Its protonated form has a pKa of 4.6, so below this value the alanine gets protonated.[15] In order to lower this pKa even further, one could attach electron-withdrawing groups to the phenyl ring. This will make it even harder for the aniline to keep its proton, so this will open the channel at even lower pH.

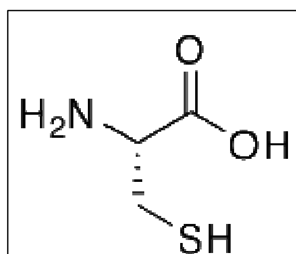

Figure 10: Structure of cysteine as a free amino acid, not incorporated in a sequence.

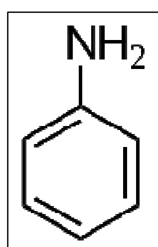

Figure 11: Structure of aniline in its neutral form.

2.2 Regulation of pH in chemical reactions

One can also think of using the M2 channel for other purposes. For example it could be used in organic chemistry. In some reactions, like the formation of an aldehyde from a chromic ester (Fig 12), acid is formed during the reaction.[16] When one wants to do a next step after this reaction in a neutral environment, this is a problem. Nowadays the H^+ ions are removed from the solution by adding a base or the product is purified before continuing the reaction. To make this easier, the H^+ ions could be removed from the solution using the M2 channel. This way, purification of the product would no longer be necessary and this is a huge gain in a multiple step reaction. Furthermore, it could also facilitate so-called 'on-pot-synthesis' because the reaction compounds could stay in the same jar all the time.

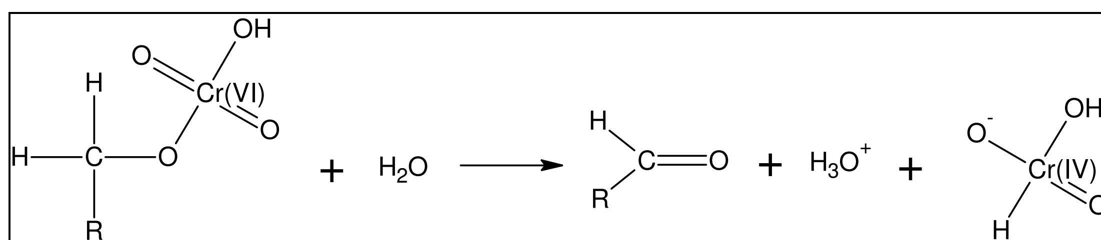

Figure 12: Formation of an aldehyde from a chromic ester. In this reaction acid is formed.

For this purpose, M2 channels could be embedded into an impermeable membrane that then would function as a selective filter for H^+ ions. If the filter is placed in the bottom of the flask in

which the reaction is performed (Fig 13), the H^+ ions could be removed at the bottom real-time. When the pH of the reaction mixture drops below a certain value, the channels would open and the H^+ ions would be caught below the membrane.

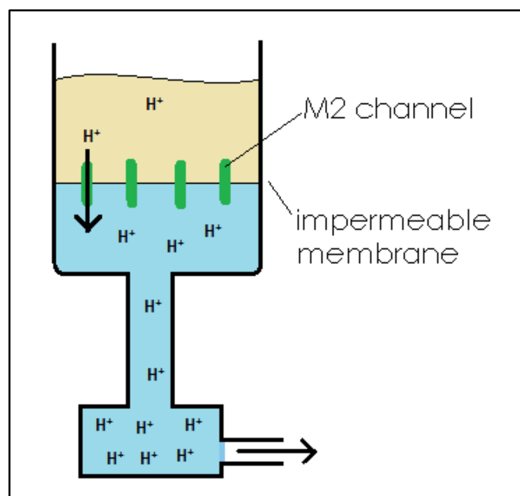

Figure 13: M2 channels incorporated in a membrane, which will act as a selective H^+ filter. The reaction mixture is positioned on top of the membrane (yellow) and the protons are captured below (blue). M2 channels are indicated green.

M2 channel in membranes could also be used to keep the pH at a certain value ranging from acidic to basic conditions. Besides in pure organic chemistry, this could be useful in enzymatic reactions. Enzymes require a certain pH at which they work the best, for instance lipase and sacharase, which have pH optima of 8.0 and 7.0, respectively.[17] The reactions can be performed in the same kind of setting as is drawn in Figure 13. Again when the pH drops below a certain value, the H^+ ions will be transferred via M2 channel into the compartment below the membrane.

Reactions that are in chemical equilibrium could be another area where M2 channel could be valuable. In the case of a reaction in which acid is formed, but the equilibrium lies towards the side of the starting compounds, the M2 channel can be used to remove the H^+ ions from the reaction mixture, so that the product will actually be formed and the reaction will not go backwards. A way of achieving this is shown in Figure 14. In this example a reaction A to B takes place in a particular solvent (pink). If the R-group is quite long, compound B will not dissolve in water. Therefore, if the H^+ could be caught, compound B would get a negative charge (C) and consequently be soluble in water (blue). This way, the compound C would be trapped in the water and could be purified right away, because the rest of the reaction compounds would stay in the organic solvent.

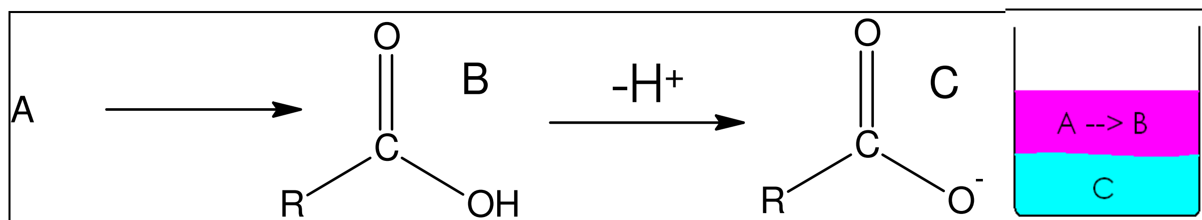

Figure 14: Reaction in which the conversion of A to B takes place in one solvent and by removing protons compound C will be trapped in the other solvent.

For the applications of M2 channel in extreme environments, such as very high or low pH, the channel should be embedded in a material that is stable in the application conditions. In stead of lipid membranes, pH-resistant support materials should be used. For example polymers could be a good choice, because synthetic polymers can be designed in many different ways. They can be made hydrophilic or hydrophobic and resistant to very acidic or basic conditions.

Conclusion

Here, a natural channel protein is discussed, which has important potential applications in nanotechnology. M2 channel offers an alternative solution for intra- and extra-cellular delivery of drugs that are either very sensitive to environmental conditions or difficult to deliver with conventional delivery methods. These channels also have the potential to be used in chemical reactions for pH regulation, reaction thermodynamics and separation of products. To the best of my knowledge none of these nanotechnological applications have yet been exploited by any research group.

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